Tall Fescue Endophyte Detection: Commercial Immunoblot Test Kit Compared with Microscopic Analysis

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ABSTRACT

A reliable, efficient, and accurate detection method for presence of the endophytic fungus *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin comb. nov. in tall fescue (*Festuca arundinacea* Schreb.) seed and plant tissue would be beneficial for tall fescue breeding and seed lot analysis. This experiment was conducted to determine the accuracy, reliability, and reproducibility of the Phytoscreen *Neotyphodium* immunoblot detection kit (Agrinostics Ltd. Co., Watkinsville, GA). Tissue immunoblot was compared with histological staining followed by microscopic analysis on tall fescue tillers from a greenhouse grow-out test, field grown spaced plants, established field paddocks, and different tall fescue seed lots. Endophyte-infected and endophyte-free tall fescue populations were evaluated by both methods. Results obtained by both methods were similar regardless of the infection level of the population, type of tissue assayed, or the technician that conducted the assay. The immunoblot detection kit was accurate and reliable and readily accommodated large numbers of samples.

THE PRESENCE of ergot alkaloid-producing endophyte (*N. coenophialum*) in tall fescue may be viewed as a positive or negative attribute, depending upon whether the fescue is used for turf or forage. Endophyte-infected forage germplasms that are non-toxic (producing little or no ergot alkaloids) are being produced (Adcock et al., 1997; J.H. Bouton, 1998, personal communication). Knowledge of endophyte infection status of seed lots and breeding populations of tall fescue is important. Seed testing services currently use histologically stained plant tissue for microscopic analysis (Clark et al., 1983). Histological staining procedures are tedious, time consuming, and difficult to use for large numbers of samples. A rapid, inexpensive, and reproducible technique is needed to evaluate tall fescue for the presence or absence of the endophyte.

Enzyme linked immunosorbent assays (ELISA) have been used to detect or quantitate endophyte in tall fescue seed and leaf sheath tissue (Johnson et al., 1982; Musgrave et al., 1986; Reddick, 1988; Reddick and Collins, 1988; Hiatt et al., 1997; Hiatt and Hill, 1997). The ELISA methods are sensitive, specific, consistent, and capable of performing analyses on large numbers of samples. The ELISA procedures, however, require expensive laboratory equipment and specialized preparation to perform the analysis.

A tissue print immunoblot technique for the detection of *N. coenophialum* mycelial proteins was first proposed by Gwinn et al. (1991) using polyclonal antiserum produced in rabbits. This technique worked equally well with either seed or plant tissue but seed analysis was more tedious and time consuming. Seed had to be scarified, allowed to imbibe water overnight, and split longitudinally prior to immunoblot analysis. Assays using this technique produced comparable results to ELISA and did not require specialized equipment to conduct the analysis.

Polyclonal antibodies are found in the immunoglobulin fraction of serum, which is composed of an almost infinite array of molecules of varying affinities and quantities. Over 90% of the Ig molecules present in the serum have little or no affinity for the target antigen (Roitt, 1994). In contrast, monoclonal antibodies are produced by a single hybridoma and all have the same structure, affinity, and specificity to a given epitope. Hence, a monoclonal antibody-based immunoblot technique has the advantages over a polyclonal antibody-based technique of greater specificity and defined affinity (Hiatt et al., 1997).

Private seed testing laboratories and commercial tall fescue breeders currently use histological staining followed by microscopic analysis to determine endophyte infection status (S. Davidson, 1996, personal communication). *Neotyphodium*-specific polyclonal antisera is not available to the private sector, therefore ELISA techniques and tissue immunoblot techniques are also not available. A monoclonal antibody-based tissue immunoblot technique requiring little immunological training is now available to public and private scientists and seed analysts. It is marketed in a kit format and can be used on seed, greenhouse-grown grow-out seedlings, and field-grown plants.

The purpose of this study was to compare the reliability, reproducibility, and accuracy of a commercially available, monoclonal antibody-based immunoblot detection assay with microscopic analysis for presence of endophyte in (i) greenhouse-grown tall fescue seedling plants, (ii) field-grown tall fescue plants, and (iii) tall fescue seed.

MATERIALS AND METHODS

Detection of Endophyte in Greenhouse-Grown Seedling Tall Fescue Plants

Seeds of endophyte-infected and endophyte-free 'Jesup Improved' tall fescue were planted into a commercial potting soil in flats containing 72 cells (each cell = 35 by 35 by 60 mm; Landmark Plastic Corporation, Akron, OH). One hundred plants were harvested each week after emergence, for 8 wk (50% of seed emergence = Day 0). Plants were sampled

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Published in Crop Sci. 39:796–799 (1999).

Abbreviations: ELISA, enzyme linked immunosorbent assay.

for immunoblot detection by cutting a single tiller at the soil surface, cutting a 1- to 2-mm cross section from the base of each tiller, and placing the cross section, with the cut end down, on a piece of nitrocellulose membrane. The membrane was placed on a cellulose sponge saturated with an extraction buffer and analyzed for endophyte presence with the Agrinostics Phytoscreen *Neotyphodium* immunoblot test kit. The remainder of the tiller was numbered, placed individually in a plastic sampling bag (Fisher Scientific, Pittsburgh, PA), and stored at −20°C until it was analyzed for the presence of endophyte by histological staining as described by Clark et al. (1983). Microscopic analysis was only performed on tillers that were large enough to provide a leaf sheath of sufficient size to perform a histological stain. The tillers were kept separate to ensure that both methods were performed on the same tiller, thereby removing any confounding due to sampling. A Student's *t*-test was used to compare the percentage of endophyte-infected plants obtained by both methods using data from Weeks 4 through 8 (Little and Hills, 1978). Data from previous harvests (Weeks 1–3) were not compared because plants were too small for microscopic analysis.

Detection of Endophyte in Field-Grown Spaced Plantings of Tall Fescue and in Pastures

Three hundred sixteen Jesup Improved endophyte-infected tall fescue plants were space planted at the University of Georgia Plant Sciences Farm located near Bogart, GA, in the fall of 1995. The soil was a Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludult). In April, 1996, individual tillers were collected over 6 sampling days until all plants were sampled. Tillers from spaced plants were evaluated for endophyte presence as described previously with the immunoblot test kit and microscopic staining. Tillers from field plants were handled in a similar manner to those grown in the greenhouse. All tiller cross sections were placed onto nitrocellulose membranes as described previously. After immunoblotting, tillers were maintained individually at −20°C until histological staining and microscopic analysis was performed. A paired *t*-test was used to compare the number of endophyte-infected tillers obtained by both methods for field-grown space plantings.

Also in April, 1996, 24 tillers were collected from each of nine 0.6-ha tall fescue paddocks located at the USDA-ARS J. Phil Campbell, Sr. Natural Resources Laboratory in Watkinsville, GA. The soil was a Cecil sandy clay loam. Two paddocks contained Jesup Improved endophyte-free and two contained Jesup Improved endophyte-infected tall fescue. The remaining five paddocks were comprised of 'Kentucky 31' tall fescue of varying endophyte infection levels. Tillers from paddocks were tested in the same manner as tillers from the spaced plants. A paired *t*-test was used to compare the number of endophyte-infected tillers obtained by both methods for the five Kentucky 31 paddocks. The Jesup Improved endophyte-infected and endophyte-free paddock means were compared by ANOVA. Infection percentage was the dependent variable for the ANOVA analysis and treatment variables were assigned to a split plot model. Method of endophyte detection was the whole plot and a factorial between infection rate and replication were the subplots. Means were separated by a Fisher's Protected LSD at the 0.05 level of probability.

Detection of Endophyte in Tall Fescue Seed

The presence of the endophyte in seed was also determined with both the immunoblot assay and histological staining for microscopic evaluation. Seed samples from six tall fescue seed populations (designated: Jesup Improved E+ GH, Jesup Improved E− GH, Jesup Improved E+ M2-5, Jesup Improved E− LS-6, 'GA5' P67, and GA5 Riddell) were analyzed. Seed used in the immunoblot were first soaked in 1.25 *M* NaOH for 1 h and rinsed with copious amounts of water. After draining excess water, seed were individually placed onto a piece of nitrocellulose membrane supported by a sponge in extraction buffer as previously described. Differences among detection methods for number of infected seed were compared by a paired *t*-test.

In a second test, five seed lots from four tall fescue popula-

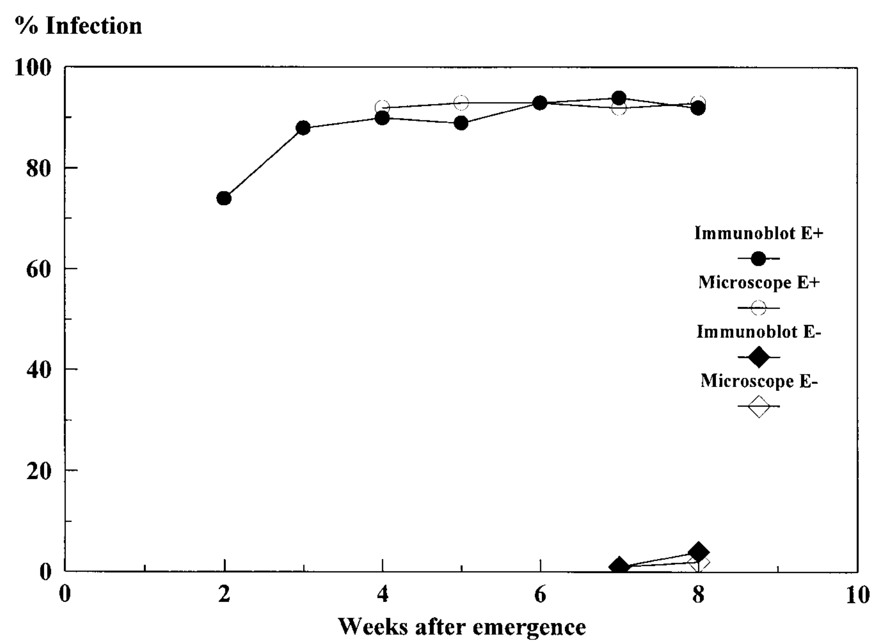

Fig. 1. Phytoscreen *Neotyphodium* immunoblot assay vs. microscopy for the determination of endophyte presence in Jesup Improved endophyte-infected (E+) and endophyte-free (E−) tall fescue populations over an 8-wk greenhouse grow-out study. Endophyte-free values were not plotted unless a positive test was recorded in at least one tiller.

Table 1. Phytoscreen *Neotyphodium* immunoblot assay compared with microscopy for the determination of endophyte presence in tillers from field grown tall fescue genotype Jesup Improved.

	Detection method†			
Sampling day‡	Immunoblot positive	Microscopy positive	Immunoblot negative	Microscopy negative
1	75	75	8	8
2	67	68	5	4
3	53	53	7	7
4	4	4	2	2
5	66	68	4	2
6	23	23	2	2
Total	288	291	28	25

† Differences between detection methods were not significant using a paired *t*-test ($P = 0.05$).
‡ Immunoblots performed on day of sampling and remainder of tiller stored at −20°C until microscopy performed.

tions were evaluated with the Phytoscreen *Neotyphodium* immunoblot assay to investigate the repeatability of the immunological test. One immunoblot membrane was set up for each seed lot (20 membranes total) and membranes were scored independently by two separate evaluators. One evaluator was well trained in immunoblot techniques and the second had minimal training in scoring membranes. Means of seed infection among cultivars and repeatability of the assay by separate evaluators were compared with ANOVA. The ANOVA model used percentage seed infection as the dependent variable, and split plot assignment with evaluators as the whole plot and populations assigned as a randomized complete block.

RESULTS

Detection of Endophyte in Greenhouse-Grown Seedling Tall Fescue Plants

The immunoblot test kit was able to detect endophyte presence in most infected seedling plants 2 wk after germination and in virtually all infected seedlings by 3 wk after germination (Fig. 1). Microscopic staining detected endophyte after 4 wk, but only 60 out of 100 seedling plants were of sufficient size to obtain leaf sheath samples.

Results for the tissue immunoblot procedure were similar to those of the microscopic staining procedure for dates when sampled plants were large enough to analyze with both methods (Fig. 1). Most seedling plants from endophyte-free tall fescue were negative for endophyte regardless of the testing method. Endophyte infection rates for seedling plants from endophyte-infected Jesup Improved tall fescue were similar with both methods. The calculated *t*-values comparing the two methods of analysis for endophyte-infected and endophyte-free Jesup Improved were 1.04 and 0.46, respectively, below the tabular *t*-value of 2.78. Therefore, the infection rates were not different regardless of which method was used to test for endophyte in the seedling plants.

Table 2. Phytoscreen *Neotyphodium* immunoblot assay compared with microscopy for the determination of endophyte presence in tillers from five Kentucky 31 tall fescue field paddocks of varying endophyte infection levels.

	Detection method†			
Paddock number	Immunoblot positive	Microscopy positive	Immunoblot negative	Microscopy negative
1	6	6	18	18
2	14	13	10	11
3	17	17	7	7
4	7	7	17	17
5	8	8	16	16
Mean	10.4	10.2	13.6	13.8

† Differences between detection methods were not significant using a paired *t*-test ($P = 0.05$).

Table 3. Mean percentage of endophyte infection in tillers from four tall fescue field paddocks, two endophyte infection levels, and two endophyte detection methods.

Paddock genotype	Phytoscreen immunoblot	Microscopy
Jesup Imp E−	0†	0
Jesup Imp E+	91.6	93.8
LSD (detection method, 0.05)	N.S.	
LSD (genotype, 0.05)	6.3	

† Values represent mean of two paddocks.

Detection of Endophyte in Field-Grown Spaced Plantings of Tall Fescue and in Pastures

Both tissue immunoblot and microscopic examination for endophyte were conducted on the same tiller to avoid confounding due to sampling error. Initially the two procedures gave different results on seven out of 316 tillers tested. These seven plants were reevaluated using a second tiller. One plant that originally tested negative with tissue immunoblot but positive by microscopy, tested positive by both methods when reanalyzed. Three tillers that originally were negative by microscopy but positive by immunoblot, were positive for both methods upon reanalysis. Three tillers that were positive by microscopy, but negative by immunoblot, retained their respective positive and negative results upon reanalysis (Table 1). When using different sampling days as replications, the calculated *t*-value (1.46) comparing the two methods was less than the tabular value (2.57) suggesting the two methods gave similar results.

Kentucky 31 tall fescue paddocks with varying infection rates were tested for endophyte presence with the two methods. The two methods detected identical infection rates with the exception of one paddock, where microscopic staining determined one less tiller to be infected than did the immunoblot procedure (Table 2). The *t*-value from the paired *t*-test was 1.00, below the tabular value of 2.78. Therefore, the two endophyte assays detected similar rates of endophyte infection.

Table 4. Phytoscreen *Neotyphodium* immunoblot assay compared with microscopy for the determination of endophyte presence in seed from six tall fescue populations of varying endophyte infection levels.

	Detection method†			
Population	Immunoblot positive	Microscopy positive	Immunoblot negative	Microscopy negative
Jesup Imp E+ GH	97	96	3	4
Jesup IMP E− GH	3	0	97	100
GA5 P67	72	60	28	40
GA5 Riddell	29	25	71	75
Jesup Imp E+ M2-5	92	94	8	6
Jesup Imp E− LS-6	2	0	98	100

† Differences between detection methods were not significant using a paired *t*-test ($P = 0.05$).

Table 5. Analysis of variance for percentage of endophyte infected seed in a repeatability study of the Phytoscreen *Neotyphodium* immunoblot assay using four tall fescue populations, two evaluators, and five replications.

Source	df	Mean squares	*F* value
Population	3	17 325.97*	2150.39
Rep	4	8.40	1.04
Evaluator	1	0.90	0.11
Population × evaluator	3	16.37	2.03

* Significant at the 0.05 probability level.

When replicated pastures of endophyte-infected and endophyte-free Jesup Improved tall fescue were tested for endophyte with the two methods, there were no significant differences in the results between the endophyte detection assays (Table 3). There was, however, an endophyte effect.

Detection of Endophyte in Tall Fescue Seed

When seed from six tall fescue populations with varying infection rates were evaluated by both endophyte detection methods, no significant differences resulted between the detection assays (Table 4). The calculated *t*-value (1.73) comparing the two techniques was less than the tabular value (2.57). Thus, the two detection methods gave similar results in tests of seed.

In analysis of variance, the only treatment variable that was significant for endophyte detection by the immunoblot assay in tall fescue seed was population (Table 5). Data for two evaluators using the immunoblot were analyzed to provide information regarding sampling error associated with replications and error associated with subjective scoring between evaluators (Table 6). The four populations used ranged in endophyte infection from 2.4 to 96.8%. Sampling error, measured as variability among replications, was similar to or greater than error associated with subjective scoring between evaluators. Therefore, the immunoblot test kit produced the same results regardless of which evaluator scored the immunoblot membrane.

DISCUSSION

The greenhouse grow-out study was designed to simulate test conditions used to determine viable endophyte in a tall fescue seed lot. The immunoblot detection system was capable of approximating maximum values of endophyte in the seedling plants 3 wk after germination, while microscopy required 6 wk until all plants were large enough to sample. This suggests the immunoblot procedure could be a time saving analytical method. However, caution should be exercised because when seed lots have been stored for extended periods, the endophyte may lose vigor within individual seed and not be expressed during the early seedling stage, thus more mature plants (e.g., 6 wk old or more) may be required to obtain accurate endophyte viability data (Welty et al., 1987).

Table 6. Mean percentage of endophyte infected seed in a repeatability study of the Phytoscreen *Neotyphodium* immunoblot assay using four tall fescue populations, two evaluators, and five replications.

Population	Evaluator	Percent infected	sd_s†	sd_t‡
GA5 P67	1	72.8	3.03	1.92
	2	71.6		
GA5 Riddell	1	30.0	2.27	2.61
	2	28.4		
Jesup Imp E+ M2-5	1	92.8	3.79	2.19
	2	96.8		
Jesup Imp E− LS-6	1	2.4	2.46	0
	2	2.4		

† sd_s = Standard deviation between replications (sampling error).
‡ sd_t = Standard deviation between evaluators (technician error).

Regardless of whether the tissues tested were seeds, tillers from seedling plants, or tillers from mature field-grown plants, and regardless of the varieties tested, the immunoblot assay and microscopy gave similar infection rates. We found the immunoblot technique amenable to working with large numbers of samples and unlike current polyclonal immunoblot techniques the Phytoscreen immunoblot assay utilized a method to transfer endophyte protein to the nitrocellulose membrane that was similar for all types of plant material tested. Interpretation of results was also uncomplicated for those with minimal immunological training. From these experiments, we concluded that the immunoblot test kit provides a reliable and accurate assay of endophyte infections in seed, seedlings, and mature tillers of tall fescue.

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